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UREASE FROM THE LUGWORM, *ARENICOLA CRISTATA*

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Urea can arise from arginine and purine catabolism. Small amounts of urea are found in sea water and can be used by phytoplankton for growth (Mitamura and Saijo, 1975; McCarthy, 1972). In animals containing urease (urea amidohydrolase, EC 3.5.1.5), urea can serve as a source of ammonia. Although ammonia is considered an end product of nitrogen metabolism in aquatic invertebrate animals, ammonia can also play a role in ion regulation (Maetz, 1975), osmoregulation (Gilles, 1975), buffering of blood and urine (Campbell, 1973), calcification (Speeg and Campbell, 1968; Crossland and Barnes, 1974), and some developmental processes (Epel, Steinhardt, Humphreys, and Mazia, 1974).

Urease is found in some marine molluscs (Hanlon, 1975), polychaetes (Hult, 1969; Campbell, 1973; Razet and Retière, 1967), cestodes (Bishop, 1975; Simmons, 1961), and starfish (Brookbank and Whiteley, 1954). The gastric urease activity seen in vertebrates is generally associated with the microflora and is not considered to be of animal origin (Delluva, Markley and Davis, 1968; Rahman and Decker, 1966). In the medicinal leech, the low level of kidney urease activity is associated with *Corynebacterium* sp. living in the lumen fluid (Büssing, Döll and Freytag, 1953). Because the properties of bacterial ureases are varied, demonstration that the urease activity found in an animal's tissue is of animal origin is often difficult. In studies with the land snail (Speeg and Campbell, 1968), the urease was shown to be unique in that its properties differed from those of the urease of commensal microorganisms.

Except for the studies with the land snail urease (McDonald, 1970) no attempt at purification or characterization of animal ureases has been reported. Additionally, in describing the ureases from invertebrate sources, assays are rarely standardized so that a distinction between animal and microbial ureases can be made. This report describes the partial purification and characterization of lugworm (*A. cristata*) gut tissue urease and is the first step in determining the organismic origin of this important ammonia-forming enzyme.

Methods and Materials

Lugworms, *Arenicola cristata* Stimpson, were provided by the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts or were purchased from NEMSCO, Bourne, Massachusetts. All lugworms were collected in the area south of Cape Cod and were held in running seawater tables for several days prior to use.

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TABLE I

Partial purification of urease from A. cristata *gut.*

Preparation	Volume (ml)	A_{280}	Units	Total activity	Specific activity*
Dialyzed extract	62	10.6	2.4	148	0.23
DEAE-cellulose column (+)	10	11.3	10.4	104	0.92
Sephadex G-150 (+)	11	0.5	6.8	75	13.60

* Specific activity is units/A_{280}.
(+) Combined fractions.

Nessler's reagent, DEAE-cellulose, reducted glutathione and Tris (hydroxymethyl) aminomethane were purchased from Sigma Chemical Company. Reagents for scintillation counting were purchased from New England Nuclear Company. Sephadex G-50, G-150, aldolase, ovalbumin, and ribonuclease were purchased from Pharmacea Fine Chemicals. Iodoacetamide, acetohydroxamic acid, N-ethylmaleimide, and hydroxyurea were purchased from ICN Pharmaceuticals, Incorporated and prepared in ice water just before used. [^{14}C] urea with a specific radioactivity of 55 mCi/mmole was purchased from Schwarz-Mann. The [^{14}C] urea was diluted with [^{12}C] urea to a specific radioactivity of approximately 0.5 μCi/μmole. All other reagents were reagent grade from Fischer Scientific Company or Baker Chemical Company. All pH measurements were made at 22° C with a Beckman Zeromatic pH meter. Unless otherwise stated, all reagent solutions were prepared in distilled-deionized water.

During purification, the enzyme was assayed using the previously described procedure (Bishop, 1975) at pH 7 in 0.1 M potassium phosphate with 20 mM urea at 30° C. Ammonia formation was determined by Nesslerization after diffusion. A radiometric assay (McDonald, Speeg, and Campbell, 1972) was used for all kinetic and inhibitor studies. For the radiometric assay, the enzyme was incubated with the [^{14}C] urea solutions in Erlenmeyer flasks (25 ml) closed with gum rubber stoppers equipped with polyethylene cups containing 0.2 ml of 1 M hyaminehydroxide in methanol. Reactions were terminated by addition of 1 ml of N H_2SO_4 and the evolved CO_2 trapped in the hyaminehydroxide. Ten μmoles of $NaHCO_3$ were added to the reaction mixture as carrier for the [^{14}C]CO_2 in all the radiometric assay incubations. After shaking for 1 hr at room temperature, the cup was transferred to a scintillation counting vial containing 15 ml of toluene which contained 5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis [2-(5-phenyloxazolyl)] benzene per liter. Radioactivity was determined using a Beckman LS 230 liquid scintillation spectrometer. Correction for self absorption was made using the external standard method.

Protein was estimated by determining A_{280} using a Guilford Spectrophotometer. A unit of enzyme activity was the amount of enzyme required for conversion of urea to a μmole of ammonia/hr under the conditions of assay as described above. Specific activity was units/A_{280} of enzyme.

The Sephadex G-150 and DEAE-cellulose was prepared and the columns packed as described previously (Bishop, Barnes, and Kirkpatrick, 1972). The Sephadex G-150 column was calibrated for the estimation of molecular weights

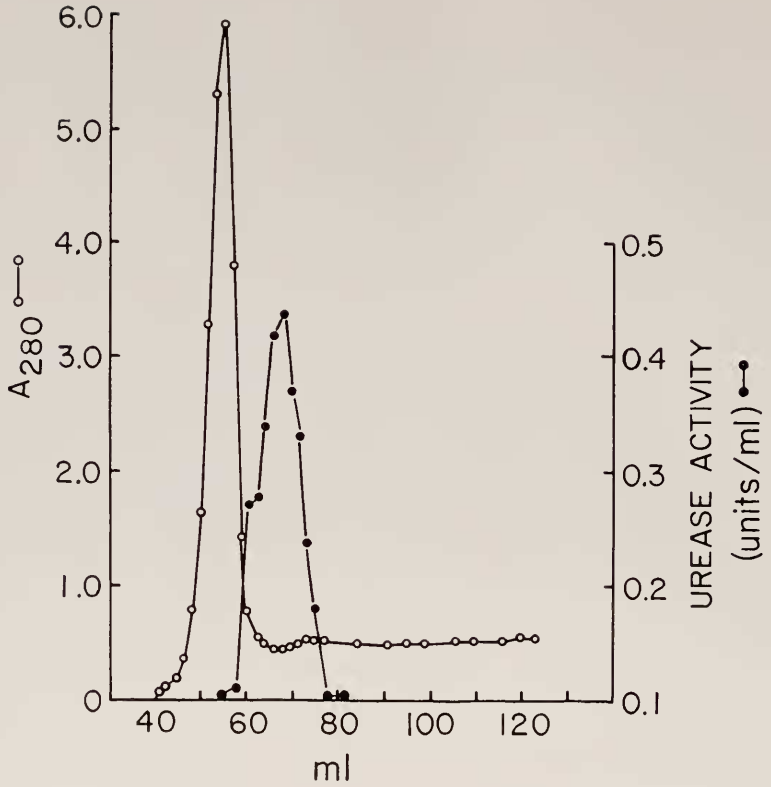

FIGURE 1. Chromatography of urease from *A. cristata* gut tissue on Sephadex G-150. Conditions are described in text. Horizontal axis describes ml of elutant and vertical axis describes absorbancy at 280 nm of the elutant. A_{280} and urease activity are indicated by the open and closed circles, respectively.

as described by Andrews (1964). Polyacrylamide gel electrophoresis was performed as described by Davis (1964) using Tris-glycine buffer at pH 8.3. No stacking gels were used and the gels were pre-electrophoresed to remove persulfate. Electrophoresis of the enzyme preparation was performed at 4° C. Staining was with Coomasse Blue.

RESULTS

During preparation of the enzyme, all procedures were performed at 0–4° C. Centrifugations were for 15 min at 15,000 rpm using an SS-34 rotor in a Sorvall RC-2B refrigerated centrifuge. Gut tissue behind the pharynx was removed from specimens of *A. cristata* and the tissue was washed thoroughly with running sea water. The tissue was homogenized in 9 ml of 0.05 M Tris-hydrochloride (pH 7.3)-1 mM reduced glutathione per gram of tissue in a ground glass homogenizer. After centrifugation the supernatant fluid was decanted and dialyzed twice for 4–8 hrs against ten volumes of 5 mM Tris-hydrochloride (pH 7.9). After dialysis, the

preparation was passed through a DEAE-cellulose column previously equilibrated with the same buffer. In a typical preparation, about 5 grams of tissue were used and the DEAE-cellulose column had a 2 × 5 cm packed wet bed volume. After addition of the enzyme, the DEAE-cellulose column was washed with 100 ml of buffer from the second dialysis. The column was then washed with 0.05 M Tris-hydrochloride (pH 7.3)-0.2 M KCl. The urease activity eluted with the solvent front and was collected in test tubes. Tubes with urease were combined and the protein concentrated by addition of 5.5 g $(NH_4)_2SO_4$/10 ml of solution. The precipitated protein was collected by centrifugation and dissolved in 2 ml of 0.05 M Tris-hydrochloride (pH 7.3)–0.2 M KCl. This preparation was applied to a Sephadex G-150 column (2 × 50 cm), previously equilibrated with the same buffer, and the column was washed at a flow rate of 12 ml/hr. The enzyme activity eluted in a symetrical peak just behind a large A_{280} peak at the void volume (Fig. 1.). The elution profile from the Sephadex column was calibrated by applying 1 ml of a buffer solution containing 4 mg each of adlolase (160,000 MW), ovalbumin (45,000 MW) and ribonuclease (13,700 MW). The apparent molecular weight of the *A. cristata* gut urease was 200,000. When Sephadex G-150 chromatography was repeated using sodium phosphate (50 mM; pH 7)-sodium acetate (100 mM), the same molecular weight was obtained. The purification procedure resulted in an enrichment of 70 fold in specific activity with a 50–60% recovery of the original urease activity (Table I).

Approximately 40 μg of partially purified urease was mixed in 25% glycerol in 0.02M Tris-glycine (pH 8.3) containing a trace of Bromphenol Blue. This mixture was applied to a 6% polyacrylamide gel column (0.7 × 8 cm) and the proteins separated by electrophoresis using 150 volts and 12 milliamps/tube. After tracking dye reached the anodal end of the gel column, the gel was removed and sliced

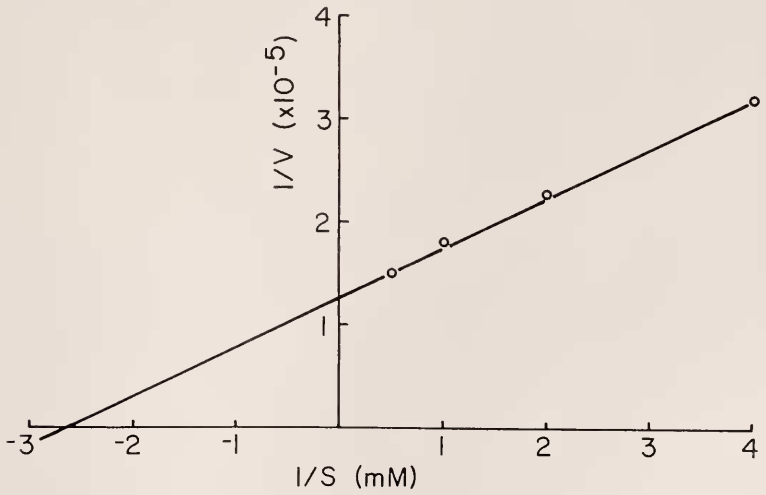

FIGURE 2. Variation in the velocity of *A. cristata* gut urease reaction with urea concentration. The reaction mixture contained the indicated amount of [^{14}C] urea (0.5 μCi/angle) 100 μmoles of potassium phosphate (pH 7.1), and enzyme in 1 ml using the radiometric assay. All incubations were at 30° C for 20 min.

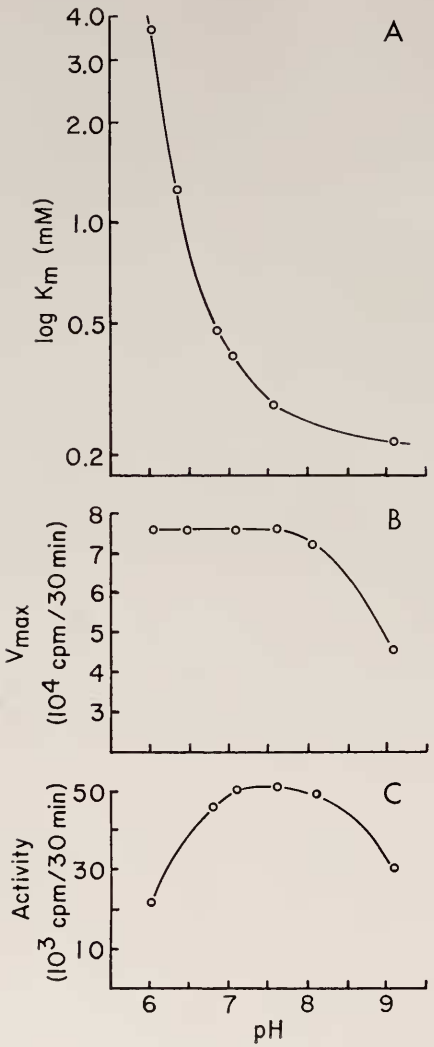

FIGURE 3. Variation in the activity of urease from *A. cristata* gut tissue with change in pH. Horizontal axis describes the pH and the vertical axis describes the activity or K_m depending upon the figure. The buffer contained 0.2 M Tris and 0.2 M KH_2PO_4 mixed in equal portions and adjusted to the appropriate pH with HCl or KOH. Each point in Figure 3A and 3B was determined from the intercepts of double reciprocal plots (Fig. 2) at the indicated pH using the radiometric assay. The reaction mixture in Figures 3A and 3B contained 100 μmoles of the Tris-phosphate buffer at the appropriate pH, between 0.2 and 5 μmoles of [^{14}C] urea (0.5 μCi/μmole), and enzyme in 1 ml. In Figure 3C, the reaction mixture contained 2 μmoles of [^{14}C] urea (0.5 μCi/μmole), 100 μmoles of Tris-phosphate buffer at the appropriate pH and enzyme in 1 ml. All incubations were at 30° C.

longitudinally. One longitudinal piece was stained with Coomassee Blue. The other piece was sliced in 0.5 cm cross sections and each section assayed for urease activity using the radiometric assay. After destaining, at least four weakly staining

blue bands and three major bands were evident. Urease activity was associated with a major band at R_f 0.35.

The partially purified preparation which had been tested for purity by polyacrylamide gel electrophoresis was used for the kinetic and inhibitor studies reported below. The enzyme could be frozen and thawed or freeze-dried and dissolved without loss of activity. The partially purified enzyme preparation was routinely stored at −20° C. Enzyme preparations held in solution in phosphate buffer (pH 7) for 2 weeks lost 40–60% of their activity. All activity was lost if the enzyme was boiled for 2 min. In the crude state, no activity was lost when held at 52° C for 3 min. When tissue homogenates prepared in Tris buffer containing 0.8 M sucrose or 0.5 M KCl were centrifuged, all urease activity was detected in the supernatant fluid fraction. The tissue activity of the enzyme averaged within 10% of 30 units/g wet weight in five preparations.

In preliminary studies, [^{14}C] CO_2 liberation from [^{14}C] urea increased linearly with increased incubation time to 40 min and in proportion to the amount of enzyme added to the reaction mixture. The pH for maximal activity was between pH 6.5 and pH 8. Using the radiometric assay, the urease activity showed saturation kinetics with respect to the urea concentration. The apparent K_m for urea was 0.38 mM in potassium phosphate buffer at pH 7.1 (Fig. 2). The K_m's for urea at pH 7.5 in 0.1 M Tris-acetate, potassium phosphate, or Tris-phosphate were all

TABLE II

Inhibition of urease from A. cristata *gut tissue. The reaction mixture contained the indicated amount of inhibitor, 100 μmoles Tris-acetate pH 7.5, 25 μg of protein, and 2 μmoles of [^{14}C] urea (0.5 μCi/μmole). Enzyme was prepared by passage of 5 mg of the partially purified preparation through a Sephadex G-50 column (40 × 2 cm) equilibrated with Tris acetate (0.02 m) pH 7.5 to remove glutathione and phosphate. In the preincubated samples, the mixture was incubated for the indicated time period without the [^{14}C] urea and the reaction started by addition of the [^{14}C] urea. In the preparations with no preincubation, the reaction was started by addition of the enzyme. The percent inhibition was determined by difference from complete reaction mixtures after a 30 min incubation using the preparations containing no added inhibitors. All incubations were at 30° C.*

Inhibitor added	Concentration mM	Per cent inhibition		
		With preincubation	(min)	No preincubation
None		0	(15)	0
Iodoacetamide	1.0	0	(15)	0
$AgNO_3$	1.0	100	(15)	100
	0.1	97	(15)	97
N-ethylmaleimide	1.0	89	(15)	67
	0.1	42	(15)	22
Hydroxylamine	1.0	93	(15)	33
	0.1	22	(15)	7
Hydroxyurea	1.0	97	(15)	32
	0.1	26	(15)	0
	0.1	49	(30)	0
Acetohydroxamate	5.0	100	(30)	96
	1.0	98	(30)	77
	0.1	98	(30)	22

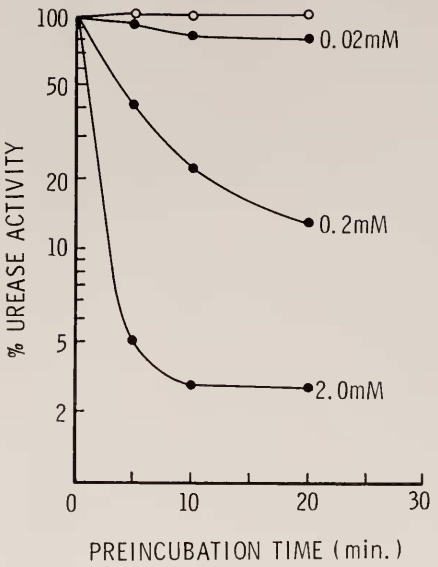

FIGURE 4. Inactivation of urease from *A. cristata* gut tissues by acetohydroxamate. The reaction mixture contained the indicated amount of acetohydroxamate (closed circles), 100 μmoles of potassium phosphate (pH 7.3), and enzyme in a 1 ml volume. Control flasks (open circles) contained no inhibitor. After incubation for the indicated time period, remaining urease activity was determined by addition of 2 μmoles of [^{14}C] urea (0.5 μCi/mole). The enzymatic reaction was terminated after a 30 min incubation and the evolved [^{14}C]CO_2 determined as described in the text. All incubations were at 30° C.

within 10% of 0.3 mM. The variation in K_m for urea-enzyme complex was determined at pH values between 6 and 9.1 (Fig. 3). The apparent K_m decreased markedly between pH 6 and pH 7 and was lowest (0.38–0.22 mM) between pH 7 and pH 9 (Fig. 3A). The V_{max} was constant between pH 6 and pH 7.6 but decreased between pH 8 and pH 9.1 (Fig. 3B). The reaction velocities measured at various pH values and at an arbitrary substrate concentration of 2 mM (Fig. 3C) were maximal between pH 7 and pH 8 in the Tris-phosphate buffer system.

Because HCl was used to adjust the pH of the buffers between pH 7.6 and pH 6 and because of the dramatic change in K_m(urea) in this pH range, it was essential to determine whether Cl^- was a competitive inhibitor. Assays were performed between 0.25 and 2 mM urea at 135 mM NaCl or twice the maximal concentration achieved in the pH experiment (Fig. 3) in Tris-phosphate buffer (pH 7.5). Although there was some noncompetitive inhibition (10% at V_{max}), there was no change in K_m for urea.

The inhibition by some sulfhydryl reactive reagents and compounds which inhibit urease activity was evaluated to further discriminate between *A. cristata* gut urease and urease from other sources (Table II). These agents behaved as irreversible inhibitors. To determine whether they were active site directed, inhibition or inactivation studies were undertaken with enzyme incubated in the presence and absence of the substrate (Table II). Iodoacetamide did not inhibit and no protection from Ag^+ inactivation was offered by incubation with the

substrate. By contrast, substrate protection was afforded against N-ethylmaleimide inactivation. Hydroxylamine, hydroxyurea, and acetohydroxamate all inhibited the enzyme and substrate protection was observed. Acetohydroxamate was a more effective inhibitor than either hydroxyurea or hydroxylamine.

The sensitivity of the enzyme to acetohydroxamate inactivation was evaluated by incubating the enzyme with various concentrations of acetohydroxamate for various time periods. Inactivation proceeded in a nonlinear fashion (Fig. 4) with time and approached a maximum degree of inhibition which was dependent upon inhibitor concentration.

Discussion

A. cristata gut urease is a reasonably stable enzyme, exists as a single soluble entity and has unique properties which distinguish it from microbial, plant, and other animal ureases.

With jack bean urease, the pH optimum is between pH 6 and pH 8 (Blakeley, Hinds, Kunze, Webb and Zerner, 1969; Lynn, 1967; Sundaram and Laidler, 1970) and the K_m for urea is 4–6 mM between pH 5 and 7 and 2–2.5 mM between pH 8.0 and 9. The bacterial ureases appear to have more varied properties but are less well studied. With *Bacillus pasteurii* (Larson and Kallio, 1954), the K_m falls from 100 mM and 130 mM at pH 5.7 and pH 6.7 to 40 mM at pH 7.7. Urease from rumen microorganisms showed a pH optimum between pH 8 and 9 and a K_m of 1.5 mM at pH 8.5 (Rahman and Decker, 1966). In *Corynebacterium renale*, the urease had a pH optimum between pH 7–8 and a K_m of 30 mM at pH 7 (Lister, 1956). In *Proteus mirabilis*, the urease activity was greatest between pH 6 and 8.3 and had a K_m of 10 mM at pH 7 (Anderson, Kopko, Diedler, and Nohle, 1969). Essentially the same properties have been reported for ureases from other *Proteus* species (Hase and Kobashi, 1967; Magna-Plaza, Montes, and Ruiz-Herrera, 1971; Speeg and Campbell, 1968). With urease from *Aerobacter aerogenes*, the K_m for urea increases from 1.5 to 6 mM as the pH decreases from 8 to 6.5 in phosphate buffer (Kamel and Hamed, 1975). Kinetic properties of ureases from animal sources have been examined in only three instances. Ureases in cestode species (Bishop, 1975; Simmons, 1961) have K_m's between 5 mM and 15 mM at the optimal pH of 7–7.5 in phosphate or tris-maleate buffer. The snail urease, on the other hand, has a low K_m of 0.1 mM at the optimal pH of pH 8.5, (McDonald, 1970; McDonald and Campbell, 1970). The variation of K_m with pH has not been evaluated for the cestode or snail enzymes.

In general, then, the microbial and plant ureases have K_m's for urea in the 1.5–250 mM range with the lower K_m's at the higher pH's. *A. cristata* gut urease exhibits K_m's in the optimal pH 7–9 range which are one-fifth to one-eighth of the lowest reported K_m's for urease from any microbial or plant source. The K_m is very similar to that reported for the snail enzyme (McDonald and Campbell, 1970). The sharp decrease in K_m between pH 6 and pH 7 suggests that a functional group on the enzyme with a pK between pH 6 and pH 7 is involved in substrate binding (Fig. 3). The broad pH optimum with a decline in V_{max} between pH 8 and pH 9 suggests that a functional group with a pK in this range is involved in the catalytic mechanism.

With regard to mechanism, we could find no evidence for the ATP-biotin

dependent urease found in green algae and some fungi (Roon and Levenberg, 1968; Thompson and Muenster, 1971).

The inhibition by hydroxylamine, hydroxyurea and acetohydroxamate observed for the *A. cristata* gut urease (Table II) is similar to the inhibition observed with all other ureases (Blakeley, Hinds, Kunze, Webb, and Zerner, 1969; Fishbein and Carbone, 1965; Fishbein, Winter, and Davidson, 1965; Gale, 1965, 1966; Gale and Atkins, 1969; Hase and Kobashi, 1967; Kobashi, Takebe, Terashima, and Hase, 1975; McDonald and Campbell, 1970; Speeg and Campbell, 1968). From the data in Figure 4 an I_{50} of about 5×10^{-5} M can be calculated for the *A. cristata* gut urease. This I_{50} is similar to that found with the jack bean and *Proteus* urease (Blakeley, Hinds, Kunze, Webb and Zerner, 1969; Fishbein and Carbone, 1965; Hase and Kobashi, 1967) but somewhat greater than the I_{50} found for the snail enzyme (McDonald, 1970). The *A. cristata* gut urease is similar to the snail enzyme in its reactivity of sulfhydryl reactive agents (McDonald, 1970). Neither are inhibited by iodoacetamide but both are strongly inhibited by Ag^+ and N-ethylmaleimide (Table II). The jack bean urease is also strongly inhibited by N-ethylmaleimide and Ag^+ (Gorin and Chin, 1965).

The molecular weight by gel filtration is somewhat lower than the 262,000 daltons reported for the snail enzyme (McDonald and Campbell, 1970) and substantially lower than the 482,000 daltons of the α-form of jack bean urease (Blakeley, Webb, and Zerner, 1969; Fishbein, 1975). Walberg (1957) reports a molecular weight of 473,000 for *Proteus* urease. Gel-filtration experiments indicate that this high molecular weight form of urease is probably predominant in other bacterial species (Kamel and Hamed, 1975; Magana-Plaza *et al.*, 1971). However, Tanis and Naylor (1968) have reported low molecular weight forms of urease in the 230,000 MW range from *Proteus* and plant sources including jack beans. Fishbein (1969) has confirmed the existance of the 240,000 MW form of jack bean urease as one of the isozyme forms of this enzyme, but he considers the parent form to be the 482,000 MW form. Jack bean urease can form aggregates or dissociate according to ionic strength, pH, glycol concentration, and thiol concentration (Fishbein, 1975). With *A. cristata* gut urease, no evidence for isozyme forms was obtained from the gel-filtration and electrophoretic experiments.

The characteristic properties of the *A. cristata* gut urease—low K_m for urea, large variation of K_m with pH, pH optimum, molecular weight, and sensitivity to inhibitors—distinguish this urease from the urease found in cestodes, the land snail, plants and microorganisms. From this preliminary characterization study, the *A. cristata* gut urease would appear to be a unique animal urease.

Not all invertebrate animals have urease activity in their gut or other tissues and the presence of urease seems unrelated to habitat or phylogeny. Present views (Campbell, 1973) seem to favor some relationship between urease function and ammonia production. As mentioned in the introduction, ammonia can play a role in pH adjustment, ion regulation, and osmoregulation in addition to nitrogen excretion. If the urease is of animal origin, then its synthesis should be under metabolic control and may be related to the regulation of ammonia formation. For instance, in the land crab, *Cardisoma guanhumi*, the uric acid which accumulates in the hepatopancreas while the animal is on land, is systematically degraded to ammonia, carbon dioxide and glyoxylate when the animal enters the water (Gifford, 1968).

The induction or regulation of the purinolytic mechanism and urease has not been investigated in marine invertebrates. In the lugworm, the presence of urease, arginase (Bishop and Crawford, unpublished results) and the purinolytic pathway (Razet and Retière, 1967) means that the arginine and purine derivatives in the tissues can serve as sources of ammonia for ion regulation or for amino acid biosynthesis in the adjustment of the intracellular osmotic pressure during salinity changes in the seawater environment.

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Summary

Urease from animal tissues is often considered to be of microbial rather than animal origin. A determination of key properties of urease isolated from an animal tissue should permit an assessment of the origin of the enzyme. Lugworm (*A. cristata*) gut urease was purified seventy fold from tissue homogenates by chromatography on DEAE-cellulose and Sephadex G-150. The apparent molecular weight by gel-filtration was 200,000. The K_m for urea declined from about 3.5 mM at pH 6 to 0.38 mM at pH 7 then decreased with increasing pH to 0.2 mM at pH 9 in Tris-phosphate buffer. The V_{max} was constant between pH 6 and 8 then declined above pH 8. N-ethylmaleimide, $AgNO_3$ but not iodoacetamide inhibited enzyme activity. Acetohydroxamate, hydroxyurea, and hydroxylamine inhibited in the manner similar to the inhibition seen with ureases from other sources. The characteristic properties of *A. cristata* gut urease—low K_m, pattern of variation of K_m with pH, molecular weight, and sensitivity to inhibitors—distinguish this urease from urease in bacteria, plants, land snails and cestodes.

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